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Expression of *MbR4*, a TIR-NBS Type of Apple *R* Gene, Confers Resistance to Bacterial Spot Disease in *Arabidopsis*

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A single disease resistance gene candidate, *MbR4*, was isolated from the wild-type apple species *Malus baccata*. This gene was predicted to encode motifs characteristic of the Toll Interleukin 1 Receptor (TIR) - Nucleotide Binding Site (NBS) of the *R* gene. Starting with an isolated cDNA clone, genomic clones were obtained via inverse polymerase chain reaction (IPCR). The *MbR4* gene has a single open reading frame (ORF) of 2178 nucleotides, a 41-b untranslated 5' region, a 21-b untranslated 3' region, and a predicted protein of 726 amino acids (82 kDa). Its deduced amino acid sequence resembles the N protein of tobacco and the NL25 protein of potato. Ectopic expression of *MbR4* induced enhanced resistance in transgenic *Arabidopsis* plants against the virulent pathogen, *Pseudomonas syringae* pv. *tomato* DC3000. Microarray analysis confirmed the induction of defense-related gene expression in pathogen-free 35S::*MbR4* heterologous *Arabidopsis* plants, thereby indicating that the *MbR4* gene likely activates a pathogen-independent resistance pathway, rather than a gene-for-gene pathway. Our results suggest that *MbR4* plays a role in the *R* gene, and may be a source of resistance for cultivated apple species.

Keywords: apple, disease resistance gene, TIR-NBS (Toll Interleukin 1 Receptor-Nucleotide Binding Site)

Genetic engineering of the immune systems in native crop plants has become a useful tool for combining high-quality fruiting traits with disease resistance. For example, in cultivars of apple, researchers have attempted to clone resistance gene analogues (RGA) by introducing disease resistance genes (*R* genes), such as *Vf* (scab resistance), from wild relatives (Vinatzer et al., 2001; Baldi et al., 2004). This Vf gene was introduced into the cultivated apple (*Malus domestica* Borkh.) from the wild, small-fruited *Malus floribunda* 821 through an early, conventional breeding program (Crandall, 1926). However, this method, first applied in 1914, did not produce any varieties of commercially acceptable fruit trees until 1970 (Dayton et al., 1970).

Direct introduction of *R* genes has the potential to reduce this time lag considerably. However, it is difficult to test the functionality of newly introduced genes in apple directly, because its genus has a long generation time and high chromosome number (2n=34), is self-incompatible, and highly heterozygous. Therefore, as a functional test, the current study applied wild apple genes in a heterologous system using *Arabidopsis*. This research was built on reports that the *Bs2* gene of pepper and the *Pto* gene of tomato function in heterologous systems (Tai et al., 1999; Tang et al., 1999; Xiao et al., 2001, 2003). In most cases of cloning for R genes, the RGAs are isolated by designing heterologous primers in conserved regions for nucleotide-binding site (NBS) motifs (Leister et al., 1996; Yu et al., 1996; Aarts et al., 1998; Meyers et al., 1998; Lee et al., 2003). Here, we used identified RGAs of a wild-type apple, *Malus baccata*, to isolate and characterize the TIR (Toll Interleukin 1 Receptor)-NBS class of *R* gene. *M. baccata*, a species common to Korea, is one of the most disease-resistant wild-type apples; its isolated *R* gene is stably expressed in *Arabidopsis*. Our objective was to better understanding the molecular basis for disease resistance in woody plants.

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MATERALS AND METHODS

Plant Material, Bacterial Inoculation, and Disease Resistance Scoring

Young, healthy leaves of *M. baccata*, a wild apple species, were obtained from the National Horticultural Research Institute in Suwon, South Korea. These leaves were used for genomic DNA isolation and total RNA preparation. To transform *Arabidopsis*, seeds of *A. thaliana* ecotype Columbia (Col-0) were planted in moistened potting soil and reared for approximately 20 d in a growth chamber at 23°C under constant

light. Leaves from *MbR4* transgenic *Arabidopsis* plants were used for RT-PCR and microarray analysis. For large-scale inoculation of the pathogenic bacterium, we used the surfactant Silwet L-77 (Duchefa) at a concentration of 0.01% (v/v) added to a suspension (10^7 to 10^8 cfu mL^{-1}) of the virulent strain *Pseudomonas syringae* pv. *tomato* DC3000, as described by Whalen et al. (1991). After the intact leaves of whole plants were dipped in that suspension, they were scored on day 5 for the presence of necrotic or water-soaked lesions surrounded by chlorosis. To examine how growth of the pathogen could be inhibited, *P. syringae* bacterial populations were sampled by macerating five randomly selected leaf discs (0.4-cm diam) in 10 mM $MgCl_2$, then plating the appropriate dilutions on fresh agar containing rifampicin and cycloheximide.

Isolation of *MbR4* cDNA Clone and Sequence Analysis

To construct the cDNA library of *M. baccata*, we synthesized cDNA with a cDNA Synthesis Kit (Stratagen, USA), according to the manufacturer's instructions. This library was screened using *Spe*I-*Eco*RI restricted RGA fragments as a probe. The fragments were derived from the conserved NBS domain, as described by Lee et al. (2003). Positive clones were examined by dye-labeled primer and terminator sequencing, on an automatic DNA sequencer (Bionex Inc. and Core Bio System Inc., Korea). A nucleic acid homology search was performed with the BLASTX program through NCBI (http://www.ncbi. nlm.nih.gov/). The nucleotide sequences of the cDNA and genomic DNA of the *MbR4* gene were then deposited in the GenBank database under accession numbers AY363616 and AY363618. Multiple sequence alignment was performed utilizing the BCM Search Launcher ClustalW 1.8 Program (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html).

Isolation of the Full-Length *R* Gene by 5′ and 3′ Rapid Amplification of cDNA Ends (RACE) Analysis

Total RNA was isolated from 1 g of apple leaves by LiCl precipitation (Simpson et al., 1993). RACE analysis was performed with the First Choice™ RLM-RACE kit (Ambion, USA) according to the manufacturer's protocol. For the 5′ RACE analysis of *MbR4*, we used two reversely complementary gene-specific primers (outer and inner) -- OLE 2825 (5′-GAGGAGGACGATGAAGATGAAGATG-3′) and OLE 2824 (5′-TCGAGAACGAGGCTTTATCGGGGAT-3′). For the 3′ RACE analysis, two reversely complementary gene-specific primers (outer and inner) were used: OLE 2854 (5′-TAAGATGGTTGTGCTGGCATGGA-3′) and OLE2853 (5′-GTAAGTACAATGTGTGACTTGAACC-3′). The final PCR products of the 5′ RACE and 3′ RACE reactions were cloned into the pCR®2.1-TOPO vector (Invitrogen, USA), and several independent clones were analyzed.

Production of Transgenic *Arabidopsis* Expressing *MbR4*

To express the *MbR4* gene in *Arabidopsis* plants, cDNA fragments (2202 bp: +41~+2243) were produced by PCR using a set of vector primers that flanked the cDNA insert: OLE1249 (5′-GCT CAG ATC TTG TGT GGA AT-3′) and OLE1250 (5′-CTG GCG TTA CAG ATC TTA AT-3′). These were ligated into a gentamycin and kanamycin resistance-conferring binary vector, pSL5. That vector is designed to provide convenience for cloning and expression of cDNA fragments by adding the N-terminus of β-galactosidase and the multicloning site of pBluescript KS(+) to pCGN18 (which contains the 35S promoter). The vector was constructed by cloning the *Bgl*II fragment of pBluescript KS(+) into the *Bam*HI site of pCGN18. A pSL5-*MbR4* recombinant plasmid (*35S*::*MbR4*) was introduced by electroporation in the *Agrobacterium tumefaciens* strain ASE, and plants were transformed via the floral dip method (Bent, 1996). The resultant transformed seeds were grown on agar plates supplemented with kanamycin (50 μg mL^{-1}); resistant plants were then transferred to soil. Plants of the T_3 generation were used in experiments for pathogen treatment and microarray-based expression profiling analysis.

RT-PCR

Expressions of the *MbR4* gene and the PR gene in kanamycin-resistant T_3 plants were confirmed by RT-PCR analysis. To detect expression of the *MbR4* gene in the transgenic plant, we performed RT-PCR with a set of gene-specific primers, OLE3160 (5′-CGGCCTCGTCTTTCGCCAAAT-3′) and OLE3107 (5′-GGTTCAAGTCACACATTGTAC-3′). As a positive control, we also used a combination of the actin-2 primer, a forward primer (5′-GGAATTCCACCATGTTCCCA-3′), and a reversely complementary primer (5′-ATTGTCACCCGATAC-3′). To detect expression of the *Arabidopsis* thaumatin gene, forward primer OLE1190 (5′-GTGATTCATGTACGGCTGCG-3′) and reversely com-

plementary primer, OLE3315 (5'-ACGCATTACCAATCAATTAGTTGTGC-3') were used. A forward primer, OLE3317 (5'-CCAGCGAAAGGGTTCTACAC-3'), and a reversely complementary primer, OLE3316 (5'-TAGCAACTAAGATTTGCTCCAGG-3'), were utilized to detect expression of the endochitinase gene.

Gene Expression Profiling in Transgenic Plants by cDNA Microarray

A cDNA microarray containing about 700 cDNA probes of *Arabidopsis* (constructed in this laboratory) was prepared to determine changes in expression in transgenic plants. Frozen leaf samples from 35S::*MbR4* transgenic *Arabidopsis* (T_3) were homogenized with liquid nitrogen. Total RNA of 35S::*MbR4* transgenic *Arabidopsis* (T_3) was isolated by the RNeasy Plant Mini Kit (Qiagen, Germany), following the manufacturer's instructions. The RNA was labeled by directly incorporating Cy3- or Cy5-conjugated dUTP (Perkin Elmer Life Sciences, USA) during reverse transcription. The overall procedure of hybridization and washing was performed according to the protocol described at http://cmgm.stanford.edu/pbrown. After washing, the slides were immediately scanned with an ArrayWoRx scanner (Applied Precision, USA). Intensity values were quantified from the resultant pairs of TIFF files using ImaGene image analysis software (BioDiscovery, USA), and were analyzed by the GeneSight software package. Analyses were performed with mean signal intensity values assigned for each spot. Each element ratio of the mean hybridization intensity was normalized by dividing it by the mean of the selected subset (actin-2). For duplicate spots on one slide, we used the mean of both elements.

RESULTS

Isolation of *MbR4*, a TIR-NBS Type *R* Gene, in the Wild Apple Species *M. baccata*

To isolate the NBS-LRR class of the *R* gene from *M. baccata*, we constructed a cDNA library (initial pfu 1.4×10^6), then screened it with a mixed probe. The probes comprised five RGA clones that contained an NBS domain in that species. The nucleotide sequences of the RGA clones were deposited in the GenBank database under accession numbers AF516645, AF516646, AF516647, AF516648, and AF516650 (Lee et al., 2003). Nucleotide sequences of the clones were determined and analyzed using BlastX algorithms in GenBank. The cDNA clone, which contained the conserved domains of the TIR-NBS type of *R* gene, shared high sequence similarity with the TMV resistance gene *N* of tobacco and the *NL25* gene of potato. This gene, named *MbR4*, possessed a 2169-bp isolated cDNA fragment.

Structure of the *MbR4* Gene

Because this isolated *MbR4* cDNA fragment was smaller than expected, based on the hybridization signal of the *MbR4* gene located at 2.3 kb on our northern analysis (data not shown), we performed 5′ RACE and 3′ RACE analysis to obtain the entire sequence information. From these experiments, we deduced the complete transcript was 2243 nt long, a size that agreed with data from the northern blot analysis. *MbR4* consists of a 2178-b putative open reading frame (ORF), potentially encoding a 726-amino acid protein, a 41-nt 5′ UTR and a 21-nt 3′ UTR. Its amino acid sequence shows greatest homology with sequences for tobacco *N* (45% identity, 61% similarity) and potato *NL25* (39% identity, 56% similarity). The predicted protein has a putative NBS comprising P-loop, kinase 2, and kinase 3a sequences, which are predicted to bind ATP or GTP, followed by a domain with unknown function that includes sequences similar to the conserved motifs GLPL, CFLY, and MHD. This ~300-residue (ARC) region also has homology with the Apaf-1 and CED-4 activators of apoptosis in animal cells (Zou et al., 1997).

Structure of the *MbR4* Genomic Gene

Southern-hybridization analysis was performed to investigate the genomic organization of the *MbR4* gene. The several bands hybridized in each digestion represented the multi-copy number of either the *MbR4* gene or its homologous sequences in the *M. baccata* genome (data not shown). From PCR amplification with a set of primers corresponding to the outermost sequences of the full-length cDNA, the genomic fragment of *MbR4* was isolated and its sequence was analyzed and compared with that of the cDNA. A putative promoter signal was assigned, which consisted of a TATA box (-35 region), a GC box (-50 region), and a CAAT box (-68 region) (Fig. 1). The ORF contained three exons (809, 1087, and 285 b) divided by two introns (532 and 117 b). Surprisingly, Intron 1 of *MbR4* showed high similarity with that of *MdFR11* (GenBank number: AY364465), which

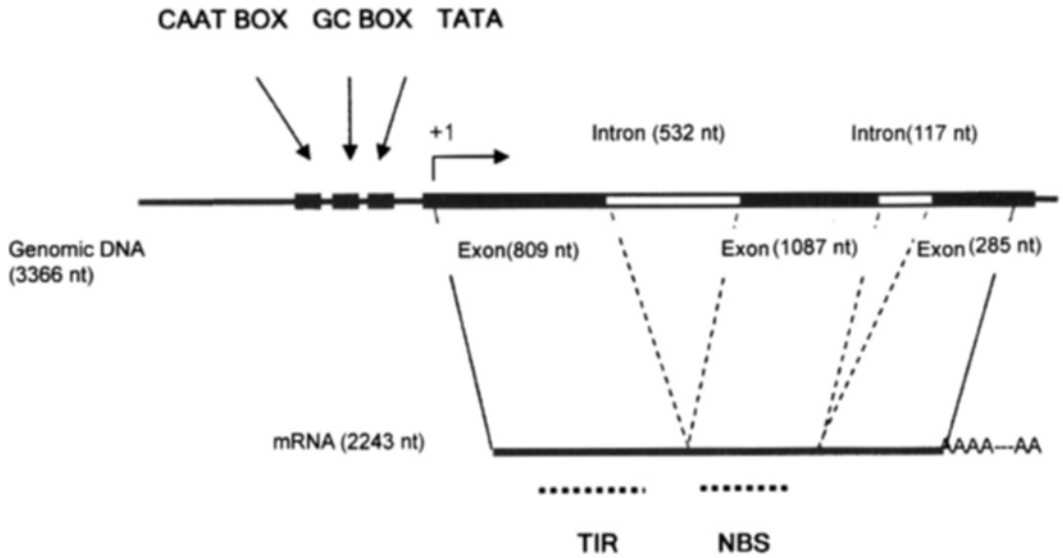

Figure 1. Schematic representation of structure for MbR4 gene. Promoter region (CAAT box, GC box, and TATA box) and exon region are shown as black boxes, while intron region, from its genomic flanking sequences, is indicated by open boxes. Conserved domains (TIR, NBS) are indicated by dashed lines.

is an *R* gene of *M. domestica* cv. *Fuji* previously identified in this laboratory (unpublished result).

Expression of the *MbR4* Gene in *Arabidopsis* Confers Resistance to *P. syringae* pv. *tomato*

To examine whether *MbR4* functions as an *R* gene, transgenic plants with the *MbR4* genes were produced. The 2202-bp-long cDNA fragments were introduced into *Arabidopsis* under the control of a viral 35S promoter, with most transgenic lines showing growth similar to the wild type. *MbR4* expression in the transgenics was confirmed by RT-PCR analysis. Specific transcript products were detected at 2.1 kb (Fig. 2B), when the primer pair OLE3160 and OLE3107 was used in the *MbR4*-transgenic lines, but those products were not found in either the wild type or the vector-only transgenic lines (Fig. 2A). All transgenic plants had been treated with the virulent pathogen *P. s. tomato* DC3000. The wild-type and the

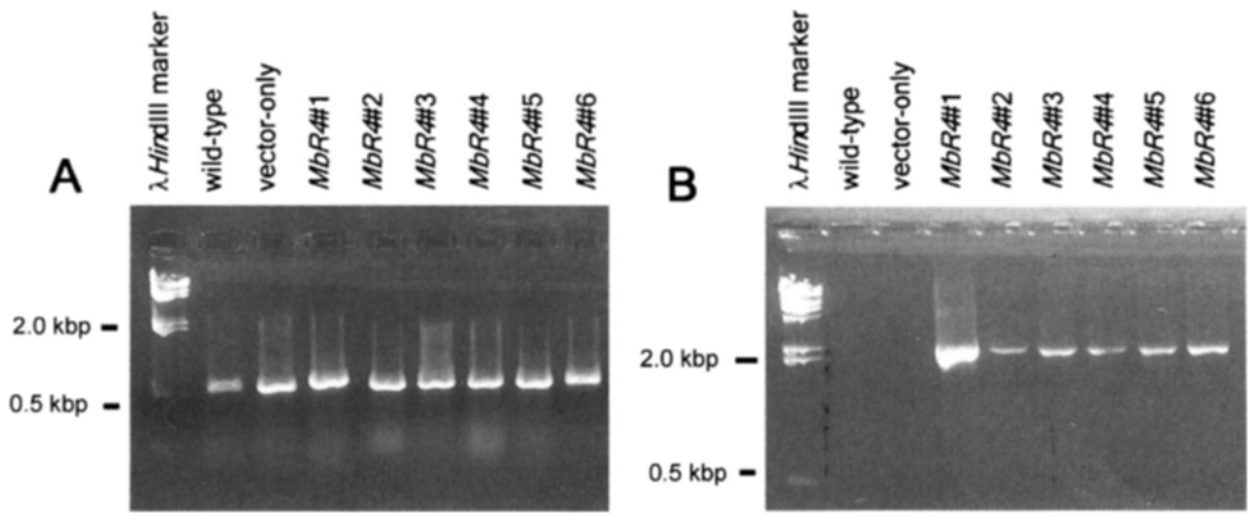

Figure 2. Overexpression of *MbR4* gene in transgenic *Arabidopsis* plants (T_3 lines). Expression of actin **(A)** and *MbR4* **(B)** genes in transgenic plants was confirmed by RT-PCR analysis, using total RNA from leaves of *35S::MbR4* transgenic plants and gene-specific primers.

vector-only transgenic plants showed strong disease symptoms, producing gray-brown leaf lesions with chlorosis that spread out from those lesions (Fig. 3A). Although mild chlorosis or necrosis occasionally developed on the leaves of the *35S::MbR4* #1 transgenic plants, the lesions with chlorosis were less dense or much smaller than those on the other two genotypes (Fig. 3B). This suggests that pathogen growth was inhibited on the transgenics. For example, the *35S::MbR4#1* transgenic plants had a 5- to 23-fold reduction in bacterial numbers (Fig. 3C) compared with the control plants ($P<0.001$). Therefore, the enhanced resistance to *P. s. tomato* in transgenic plants containing the *MbR4* gene implies that the defense mechanisms initiated by its over-expression are effective against bacterial pathogens in *Arabidopsis*.

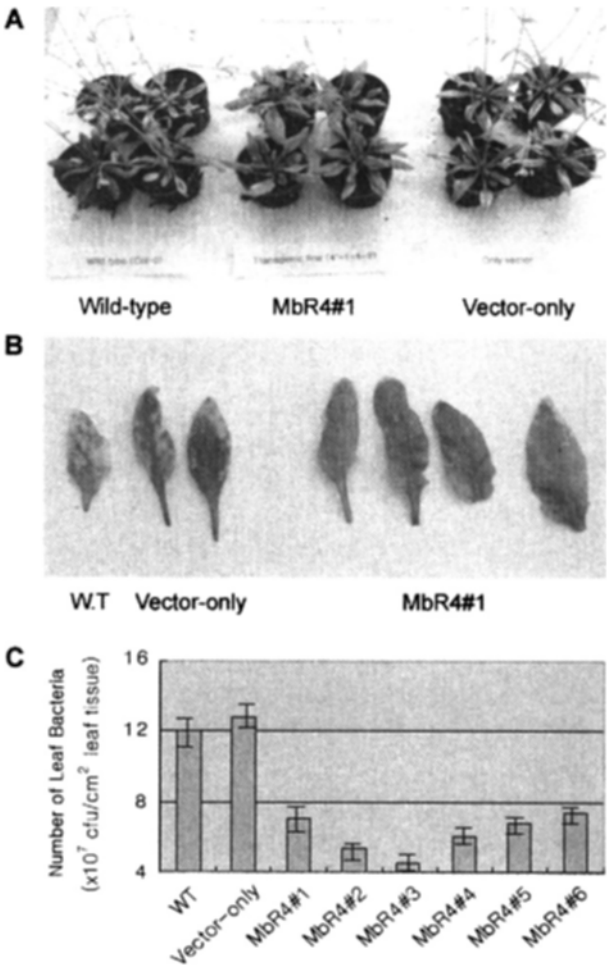

Figure 3. Analysis of disease resistance against *P. s. tomato* DC3000 strain in wild-type and vector-only as control plants, and in *35S::MbR4* transgenic plants. **A,** Resistant phenotype of *MbR4*-transgenic *Arabidopsis* and susceptible phenotype of wild-type and vector-only transgenic plants after inoculation. **B,** Disease symptoms on leaves from *MbR4* transgenic plants and wild type after inoculation. **C,** Growth of *Pseudomonas* strains in wild-type (Col-0) and vector-only (35S::pSL5), *35S::MbR4*-transgenic plants was compared by counting bacteria collected from infected leaves after 5 d. Transgenic plants were from T_3 generation. Values are average of three experiments. Control and transgenic plants differed significantly (*t*-test, $P>0.001$). Error bars indicate±SE.

Altered Expression of Plant Defense-Related Genes in Transgenic Plants

To examine changes in defense-associated and regulatory transcripts in our 35S::*MbR4* transgenic plants, microarray-based expression profiling analysis was undertaken. Correlation coefficients for the expression values from the *35S::MbR4#1* line vs. the wild type and from the *35S::pSL5* line vs. the wild type were 0.84 and 0.86, respectively, suggesting that overall expression patterns were altered in those transgenic plants. Approximately 170 defense-related genes on the microarray were further analyzed, including plant defense-related proteins such as PR5 (putative thaumatin protein), endochitinase, glycine-rich cell wall protein, glutathione S-transferase (GST30), polygalacturonase, pectinase, and beta-amylase enzyme. Out of 170 genes, 21, including those for thaumatin protein and endochitinase, were induced

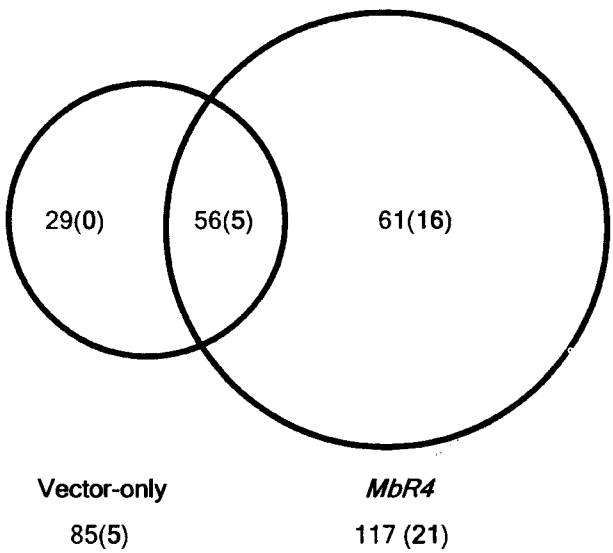

Figure 4. Expression profile analysis of over-expressed *MbR4* gene from 35S::*MbR4*-transgenic plant. Venn diagram for numbers of overlapping and non-overlapping induced genes with a minimum 2-fold ratio in each transgenic line. Bold numbers in parentheses indicate number of defense-associated and regulatory genes.

Table 1. Defense-related genes that are induced significantly (i.e., two-fold) in *MbR4*-transgenic plants.

GenBank ID	Ratio*	Gene Identity	Clone name
At2g19050	5.66	putative GDSL-motif lipase/hydrolase	G112
D17578	3.17	fatty acid desaturase in plastids	S35
AJ003135	3.11	polygalacturonase (PGA3)	G98
AAG51965	2.95	MEK kinase (MAP3Ka)	S33
At2g33590	3.85	putative cinnamoyl-CoA reductase	G23
At4g22880	3.81	putative leucoanthocyanidin dioxygenase (LDOX)	G9
AF062915	3.37	putative transcription factor (MYB90)	LIU87
AT4g36010	2.13	putative thaumatin protein	LIU45
AF043528	2.37	20S proteasome subunit PAG1 (PAG1) mRNA	L58-1
AJ003135	3.53	polygalacturonase	G99
D63460	2.81	alcohol dehydrogenase	L48-1
AF197940	4.71	SAM:phospho-ethanolamine N-methyltransferase (NMT1)	LIU123
L04173	2.99	glycine rich protein	LIU8
AF001949	2.57	homeobox-leucine zipper protein ATHB-12 (Athb-12)	LIU60
NP_564635	3.31	MEK kinase (MAP3Ka)	S17
At3g57390	2.13	MADS transcription factor-like protein agamous-like protein 15	PRU9
AF159801	2.72	lipid transfer protein 4, complete cds.	LIU19(L)
AY056244	6.15	putative glucosyltransferase	G69
AJ250341	3.38	beta-amylase enzyme (ct-bmy gene)	S46
AJ223189	4.74	PGA2	G106
AY042874	3.23	glucose-6-phosphate/phosphate-translocator precursor	G54

*transgenic/nontransgenic

Table 2. Defense-related genes that are induced significantly (i.e., two-fold) in vector-only transgenic plants.

GenBank ID	Ratio*	Gene Identity	Clone name
AY056244	2.98	putative glucosyltransferase	G69
AF159801	2.68	lipid transfer protein 4	LIU19(L)
AJ250341	2.45	glucose-6-phosphate/phosphate-translocator precursor	G54
AJ223189	2.33	PGA2	G106
AY042874	2.00	beta-amylase enzyme (ct-bmy gene)	S46

*transgenic/nontransgenic

at least two-fold in *35S::MbR4#1*, while 5 genes in *35S::pSL5* were induced by about two-fold (Fig. 4, and Table 1). Genes that were induced more than two-fold in the transgenic plants or the vector-only transgenic plants are listed in Table 1 and Table 2, respectively. Those include several PR genes, characteristically known as thaumatin protein and endochitinase, which were induced two-fold only in transgenic *Arabidopsis* plants that over-expressed *MbR4* (Fig. 5). Our observations strongly suggest that, even under pathogen-free conditions, the over-expressed *MbR4* gene regulates expression of a number of PR genes that are normally up-regulated in response to a pathogen attack.

DISCUSSION

The focus of this study was to identify a novel TIR-NBS-LRR class of *R* gene, *MbR4*, from a cDNA library of the wild apple species *M. baccata*. We also investigated the defense gene expression and resistance to pathogens in transgenic plants that over-expressed that gene. The complete transcript of *MbR4* was 2243 b long; the genomic gene consisted of 2 introns and 3 exons. Putative proteins encoded by the *MbR4* gene were most similar to the TMV virus resistance gene *N* of tobacco (Whitham et al., 1994) and the *NL25* gene of potato (Hehl et al., 1999), both of which have the same structures as TIR-NBS genes, but without the

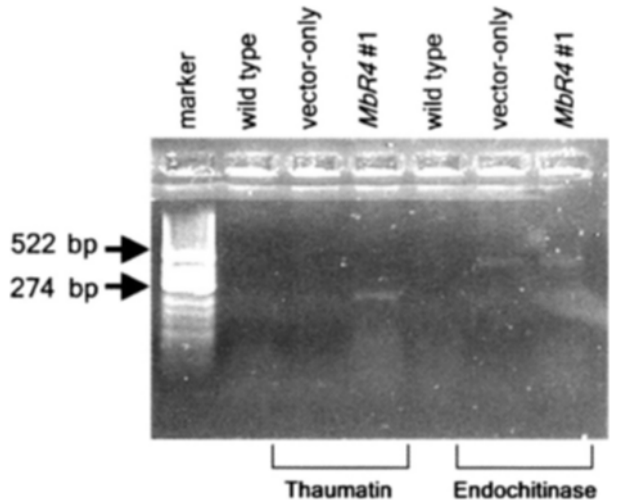

Figure 5. RT-PCR analysis of thaumatin and endonuclease transcripts in 35S::*MbR4*-transgenic *Arabidopsis* leaves. Leaves were harvested from kanamycin-resistant T_3 plants. Thaumatin transcript size is 274 b; that of endonuclease transcript is 522 b. Size of DNA molecular mass standard is indicated on the left.

LRR region. This suggests that *MbR4* might generate proteins with similar functional properties, and may include instructions to produce different proteins through differential splicing, as N_L products that occur from the alternative splicing of the *N* protein. Such alternative splicing of the intact single *R* gene is regulated by pathogen-induced signals (Dinesh-Kumar and Baker, 2000). Moreover, the TIR domain has been implicated in pathogen recognition (Luck et al., 2000). Hence, *MbR4*, with only the TIR-NBS domain, may play an important role in conferring complete resistance to a specific pathogen.

Recent agricultural trials have proven that the genetic resistance approach is an efficient way to increase crop yields. Specific *R* genes may confer either race-specific or broad-spectrum resistance. In the case of our cloned *MbR4* gene, its ectopic expression in *Arabidopsis thaliana* resulted in significantly reduced disease symptoms among plants infected by virulent *P. s. tomato* DC3000 strains when compared with wild-type controls or transgenic plants containing only the vector (Fig. 4). Moreover, the transgenic plants maintained healthy normal growth even when not exposed to the bacteria. When several *R* genes have previously been introduced into other plant species via *Agrobacterium*-mediated transformation, over-expression of some of them in heterologous plants often has either resulted in the development of necrotic symptoms in the absence of a pathogen attack or yielded plants with altered development traits, due to the finely-tuned relationship between transgenic R proteins and signaling partners in their native species (Tang et al., 1999).

Resistance in heterologous plants suggests that 35S::*MbR4* activates through a pathogen-independent resistance pathway, rather than a gene-for-gene resistance pathway. If the gene product of *MbR4* is a functional R protein in the apple, the 35S::*MbR4* transgenic plants might activate the native defense system in *Arabidopsis* by inhibiting bacterial growth. Expression of pathogen-related genes, e.g., *Pto* of tomato (Tang et al., 1999; Xiao et al., 2001, 2003; Li et al., 2002), *Bs2* of pepper (Tai et al., 1999), and *Rx2* of potato (Bendahmane et al., 2000), is either elevated or gives plants enhanced resistance to a global range of pathogens. Thus, the *MbR4* gene might also possess the capacity to defend against numerous pathogens in heterologous transgenic plants.

This hypothesis seems especially likely given the high expression level of defense-related genes in transgenic plants, as shown in our microarray experiments. The number of clones with two-fold induction ratios in 35S::*MbR4*-transgenic plants was much larger than that for vector-only transgenic plants. It is especially noteworthy that expression was three times greater than in the vector-only plants, clearly indicating that *MbR4* plays a role in plant defense. Although most *R* genes are up-regulated only when the plant is attacked by herbivores or pathogens, even without pathogen infection our *MbR4*-transgenic plants induced several significant defense genes (Tables 1 and 2), such as those involved in gene-encoding signal transduction proteins and downstream defense genes. The latter includes those encoding pathogenesis-related (PR) proteins and enzymes involved in the generation of phytoalexins, lignifications, endochitinase (Dellagi et al., 2000; Ding et al., 2002), glycine-rich cell wall protein (Xiao et al., 2001), polygalacturonase (pectinase) (Bergey et al., 1999), and beta-amylase (Dicko et al., 2001). Proteins related to fatty acid signaling and metabolism, e.g., fatty acid desaturase (Kachroo et al., 2001) and putative GDSL-motif lipase/hydrolase (Jirage et al., 1999), are also produced. Finally, as shown by our RT-PCR analysis of the thaumatin gene and endochitinase gene (encoding PR protein), the 35S::*MbR4*-transgenic plants showed high expression levels of these genes compared with the vector-only transgenic plants.

We conclude that 35S::*MbR4*-transgenic *Arabidopsis* may be a model system for inducing resistance against pathogens without any discernable inhibition

in plant growth. However, we have not yet determined whether and how the 35S::*MbR4* gene is activated in apple species. More investigation is required to develop a simple method for apple transformation and manipulation, as well as to demonstrate that the *MbR4* gene may function especially to confer apple pathogen resistance in apple species.

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